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14. ABSTRACT Heparanase (HPR1) is an endoglycosidase that specifically degrades heparan sulfate proteoglycans, a main constituent on the cell surface and in the extracellular matrix and basement membrane. The role of heparanase in breast cancer tumorigenesis remains unclear. In particular, whether HPR1 enzymatic activity is required for its stimulatory effect on tumor growth and initiation are not fully understood. Here we report that the C-terminus of HPR1, which lacks the enzymatic activity, was able to accelerate breast cancer formation in a somatic breast cancer mouse model since mice infected with RCAS-Neu virus plus RCAS-8C (a vector encoding the C terminus of HPR1), developed breast cancer faster than that infected with RCAS-Neu plus RCAS-GFP control virus. Our results suggest that HPR1 may promote tumor growth independent of its enzymatic activity.

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Table of Contents

Page

Introduction

Heparanase-1 (HPR1) is an endoglycosidase overexpressed in many malignancies including breast cancer (1; 2). Previous studies suggest that the enzymatic activity of HPR1 can promote tumor angiogenesis and growth by degrading extra cellular matrix and releasing the growth factors. Since the C-terminus of HPR1 can activate the PI-3 kinase pathway and induce endothelial and tumor cell migration independent of its enzymatic activity, it is not clear whether its enzymatic activity or C-terminus or both contribute to breast tumor initiation and growth. The goal of this project is to dissect the opposing effect of HPR1 enzymatic activity and HPR1 C-terminus epitope on breast tumor initiation in a clinically relevant mouse breast cancer model. We proposed to determine if HPR1 knockdown will suppress or accelerate breast tumor initiation mediated by three oncogenes, PyMT, Neu and Wnt, and whether HPR1 C-terminus or an enzymatically dead HPR1 can stimulates breast tumor initiation, whereas full-length HPR1 has no effect or is less effective in stimulating breast tumor initiation and progression.

Experimental procedures and results

Preparation of RCAS vectors. Three RCAS vectors containing a full-length HPR1 gene, an enzymatic activity-dead HPR1 gene (RCAS-DM-HPR1, double mutations at amino acid residues 225 & 343), and a C-terminus gene fragment (RCAS-8C, with a fusion of 8-kDa and the C-terminus of HPR1 including amino acid residues from 415-543). All inserts were tagged with a Myc epitope. This allowed us to titrate virus concentrations and monitor the expression levels in vivo. Western blot analysis with an anti-Myc tag antibody revealed that HPR1 was detected as a 18-kDa protein in DF-1 cells transfected with RCAS-8C vector, whereas the full-length HPR1 was detected as 50-kDa protein. Immunofluorescence staining revealed that RCAS-HPR1 virus-infected DF-1 cells had lower cell surface heparan sulfate levels, compared to RCAS-8C-infected DF-1 cells (. These results confirmed that the C terminus of HPR1 did not have HPR1 enzymatic activity.

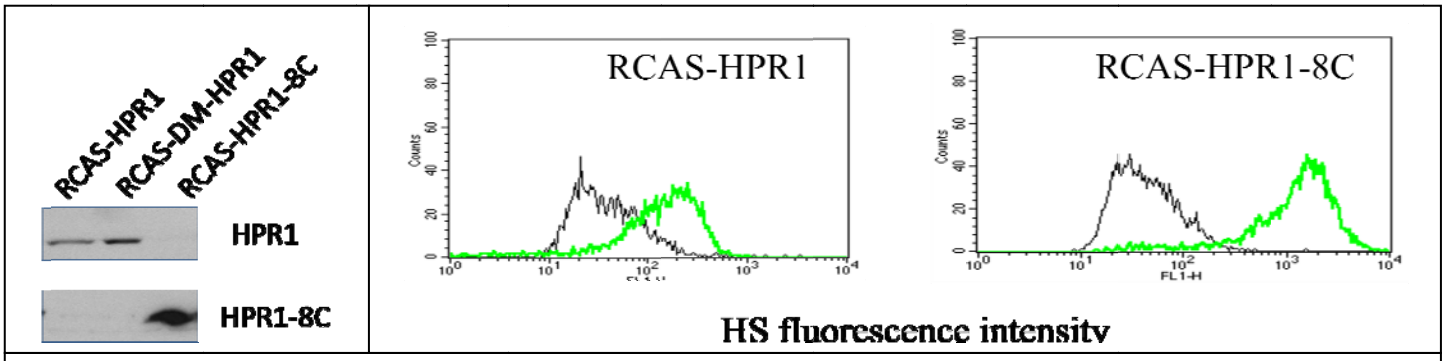
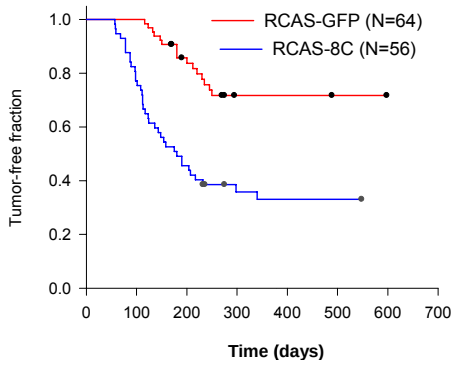


Fig. 1. HPR1 expression and cell surface HS levels. DF-1 cells were infected with RCAS-HPR1 (left), RCAS-DM-HPR1 (middle) or RCAS-HPR1-8C. After incubation for 48 hr, the cells were harvested and analyzed for HPR1 expression by Western blot with an anti-Myc epitope antibody or for cell surface HS levels by staining with an anti-HS IgM mAb followed by FACS analysis. Black line, isotype control; Green line, anti-HS IgM.

Tumor latency. We originally proposed to use TVA transgenic mice intercrossed with MMTV-PyMT, Neu, or Wnt transgenic mice. We have changed these experimental procedures by co-infecting TVA transgenic mice with RCAS vectors encoding the oncogene PyMT, Neu or Wnt plus the control RCAS vector or the vector encoding RCAS-8C. As shown in Fig. 2A, mice infected with RCAS-Neu plus RCAS-8C vectors developed breast cancer much faster than mice infected with a control RCAS-vector, the mean tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-8C were 180±30, whereas the mean tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-GFP were 480±25. These observations strongly suggested that that HPR1 enzymatic activity is not necessary for its tumor promoting effect.

Fig. 2. HPR1 knockdown delays breast cancer formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-Neu plus RCAS-HPR1-8C (blue line) or RCAS-Neu plus RCAS-GFP virus (red line) by intraductal injection of $1x10^7$ virions each. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test ($p<0.01$)



KEY RESEARCH ACCOMPLISHMENTS:

Task 2. To determine whether HPR1 enzymatic activity suppresses breast tumor initiation (Year 2)

1. Successfully cloned the C terminus of the HPR1 gene and two mutant constructs into RCAS vector, confirmed their expression and demonstrated the C-terminus of the HPR1 gene lacking enzymatic activity.

2. Successfully modified a mouse model by co-infecting TVA transgenic mice with RCAS-Neu vector plus other vector encoding tumor promoting gene, thus avoiding tedious mice strain breeding. The tumor promoting effect of HPR1 and its mutation constructs is being tested for other oncogenes.

3. Demonstrated that HPR1 C-terminus was able to promote breast cancer formation.

Research accomplished:

A. Clone HPR1, mutated HPR1 (DM-HPR1), or a DNA fragment encoding 8-kDa fused with HPR1 C-terminus epitope (8C) into RCAS vector (Q1-2) √
B. Transfect DF-1 fibroblast cell line with these vectors, collect virus, and titrate the retrovirus concentration (Western blot or IF staining with anti-Myc tag antibody) (Q1-2) √
C. Infect breast cancer cell lines (derived from TVA transgenic mice) with these retroviral vectors, expect to see increased HPR1 expression (Analyze HPR1 by Western blot and FACS analysis for cell surface HS) √
D. Breeding to produce 240 female TVA transgenic mice intercrossed with MMTV-PyMT, MMTV-Wnt, or MMTV-Neu transgenic mice (Q2-3) (changing the research plan by co-infecting TVA transgenic mice with RCAS-HPR1 virus plus RCAS-Neu, RCAS-PyMT and RCAS-Wnt onconge)
E. Induction of breast cancer by intraductal injection of RCAS virus (Table 2) (Q2-3) √
F. Analyze cell proliferation by IHC staining for BrdU, cyclin D, c-Myc (Q3-4)
G. Analyze HPR1 expression and HS by IHC & IF staining, Western blot, FACS (Q3-4)
H. Angiogenesis analysis by quantifying the number of microvessel stained with Texas Red-conjugated dextran sulfate or IHC staining for CD31 (Q3-4)
I. Whole mount to analyze tumor initiation (Q3-4)
J. Gross and histological analysis of tumor metastasis in the lungs and lymph nodes (Q3-4).

Time line

Experiments	Y1	Y2
1. To determine overall effect of HPR1 knockdown on breast tumor initiation		
A. Preparation and characterization of RCAS vector encoding oncogenes tagged with mHPR1-miRNA	Q1-2	
B. TVA transgenic mice breeding, induction of breast cancer, tumor latency	Q2-3	
C. Immunohistochemical analysis of tumor cell proliferation, angiogenesis, and metastasis	Q3-4	
2. To determine if HPR1 enzymatic activity can antagonize tumor-stimulatory effect of HPR1 C-terminus epitope		
A. Preparation and characterization of RCAS vector encoding HPR1, mutated or C-terminus HPR1		Q1-2
B. TVA transgenic mice breeding with MMTV-Wnt, Neu, PyMT transgenic mice, induction of breast cancer, and monitoring tumor latency		Q2-4
C. Immunohistochemical analysis of tumor cell proliferation, angiogenesis, and metastasis		Q3-4

REPORTABLE OUTCOMES

Manuscript: Domain-specific tumor-promoting activity of heparanase. Manuscript in preparation

CONCLUSION

We proposed to determine if HPR1 enzymatic activity can antagonize the tumor promoting effect of the C terminus of HPR1. Our studies using a syngeneic breast cancer model and somatic mouse model suggest that knockdown of HPR1 expression causes the delay of tumorigenesis and the inhibition of tumor growth, suggesting the overall role of HPR1 is to promote tumor growth and formation. Studies in the past year revealed that the C terminus of HPR1 alone was able to promote tumor initiation and growth in a somatic breast cancer model. The experiments to be conducted during the extension of this grant period will determine whether the C-terminus of HPR1 can also accelerate breast cancer formation induced by two other oncogenes, PyMT and Wnt, and whether the enzymatic activity of HRP1 will antagonize the tumor-stimulating effect of HPR1 mediated by its C-terminus.

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Domain-specific tumor-promoting activity of heparanase

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Running title: *Role of HPR1 C-terminus on breast cancer induction*

Key words: heparanase, sulodexide, TVA; Neu; PyMT;

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Abstract

Heparanase (HPR1) is an endoglycosidase that specifically degrades heparan sulfate proteoglycans, a main constituent on the cell surface and in the extracellular matrix and basement membrane. While prior studies suggest that HPR1 plays an important role in promoting tumor growth, its role in breast cancer tumorigenesis remains unclear. In particular, whether HPR1 enzymatic activity is required for its stimulatory effect on tumor growth and initiation is not fully understood. Here we report that sulodexide, a HPR1inhibitor, stimulates breast tumorigenesis and tumor growth of polyoma virus middle T antigen-induced breast tumor in a somatic breast cancer models. To further explore the role of HPR1 in breast tumorigenesis and growth, we knocked down HPR1 in a breast cancer cell line derived from mice infected with a vector encoding the Neu oncogene. We found that HRP1 knockdown led to a significant reduction of tumor growth in a syngeneic mouse model and delayed tumor growth in a somatic mouse model. To determine whether the HPR1 activity was disposable for its stimulatory effect on breast cancer formation, the C-terminus of HPR1 gene, which lacks the HPR1 activity, was cloned into a RCAS vector (designated as RCAS-8C) and tested for its potency to stimulate breast cancer formation. Mice infected with RCAS-Neu virus plus RCAS-8C developed breast cancer faster than that infected with RCAS-Neu plus a control vector encoding green fluorescence protein. Our results collectively suggest that the C-terimus of HPR1 is capable of promoting tumor growth, and its enzymatic activity is disposable for the tumor promoting activity of HPR1.

Introduction

Heparanase-1 (HPR1) is an endoglycosidase often overexpressed in a variety of adenocarcinomas [1-3]. HPR1 expression in breast cancer correlates with their metastatic potential [4, 5]. HPR1 degrades heparan sulfate (HS) proteoglycans (HSPGs), a main component of the cell surface, the extracellular matrix (ECM), and the basement membrane (BM) [6-9]. Breakdown of HSPGs in the BM and ECM leads to the release of several growth factors such as FGF and VEGF that are trapped in the tumor stroma. These growth factors can stimulate tumor angiogenesis by stimulating endothelial cell proliferation and migration. In addition, breakdown of the BM and ECM allows tumor cells to invade locally or metastasize to a distant site. HPR1 overexpression in human breast cancer cell lines or induction by estrogen led to increased angiogenesis and accelerated tumor growth in breast cancer xenograft models [10] [11] [12]. Suppression of HPR1 in a MDA-435 breast cancer cell line by ribozyme RNA or HPR1 siRNA reduced their in vitro invasive potential in Matrigel [13, 14].

In addition to its function as an endoglycosidase to cleave HS side chains, HPR1 exerts its many biological functions independent of its enzymatic activity. For example, HPR1 can enhance cell adhesion [15, 16], induce VEGF expression [17], induce tumor and endothelial cell migration, and induce Akt, p38, and Src phosphorylation [17, 18]. HPR1 can induce EGF receptor phosphorylation and stimulate tumor cell proliferation and growth in an enzymatic activity-independent manner [19]. A conservative, hydrophobic C-terminus domain of HPR1 has been recently identified to mediate these diverse biological functions [20, 21]. HPR1 C-terminus functions as a ligand to bind two potential unknown receptors (130 & 170 kDa protein) to activate the PI-3 kinase pathway [21]. More interestingly, the U87 glioma cell line overexpressing HPR1 C-terminus epitope in the absence of HPR1 enzymatic activity is more effective in stimulating tumor growth than HPR1 full molecule [21]. This raises an intriguing

possibility: how much does HPR1 enzymatic activity contribute to its angiogenic and tumor-promoting function? Two recent phase I/II clinical trials using a HPR1 inhibitor to treat patients with hepatacellular carcinoma or others types of cancer are not impressive [22, 23]. These observations suggest that HPR1 activity may be disposable for its tumor promoting activity. Here we report that using a somatic breast cancer model, HPR1 C-terminus is able to promote breast cancer formation in a somatic breast cancer model.

Materials and Methods

Plasmids. The C-terminus of the HPR1 gene (encoding amino acid 413-543) was cloned into a RCAS vector digested with a PacI and Cla I. The plasmid was designated RCAS-C. An oligonucleotide containing a Myc tag sequence (atg gaa caa aaa ctt att tct gaa gaa gat ctg) fused with a sequence encoding a 8-kDa (amino acids 36-55) of HRP1 and its complementary sequence were synthesized. The 5' end of this annealed fragment had a Not I-cleaved site, whereas its 3' end contained a cleaved Pac I site. This fragment was directly ligated into Not I/PacI-digested RCAS-C. The following plasmid designated as RCAS-8C was used to transfected DF-1 cells to generate RCAS-8C virus. To prepare miRNA vectors targeting murine HPR1 (mHPR1), three pairs of oligonucleotides with a miRNA structure that targets murine HPR1 at the nucleotide 671, 746, and 796 were synthesized and ligated into a pcDNA6.2 expression vector (Invitrogene). The effectiveness of these three miRNAs to knock down the expression of mHPR1 was analyzed for the expression of mHPR1 by Western blot and FACS analysis of cell surface HPR1 in RCAS-Neu cell line after transfection. mHPR1 miRNA insert in one construct (pcDNA/mHPR1-miRNA 746) was shuttled into RCAS-Neu vector by cloning a PCR-amplified fragment digested with Cla I and Pac I enzymes. This vector designated as RCAS-Neu/mHPR1-miRNA 746 was used to transfected into DF-1 cells. The insert of miRNA targeting β-galactosidase was PCR-amplified from a control plasmid (Invitrogen) and cloned into RCAS-Neu vector as a negative control. RCAS-GFP vector was kindly provided Dr. Y. Li (Baylor Medical College, Houston, TX).

Western blot. RCAS-Neu and DF-1 cells were harvested and lysed in Nonidet P (NP)-40 lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptinin, and 1 mM phenylmethylsulfonyl fluoride). After electrophoresis

and transfer to nitrocellulose membranes, HPR1 was detected by using a rabbit anti-HPR1 antibody, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and SuperSignal Western Pico enhanced chemiluminoscence substrate (Pierce Chemical Co., Rockford, IL). A monoclonal antibody against β-actin was purchased from Santa Cruz Biotechnology Inc., San Diego, CA.

HPR1 activity assay. Purified platelet HPR1 (50 units/μl) or serum from a pancreatic cancer patient diluted at 1:10 in HPR1 buffer (middle panel) were premixed with the indicated concentrations of HPR1 inhibitors in HPR1 assay buffer. The mixture was added to a 96-well ELISA plate precoated with Matrigel and incubated at 37°C for 16 hr. HPR1 activity was analyzed by an ELISA method according to a novel ELISA protocol established in this laboratory [24-27].

Cell proliferation assay. RCAS-Neu tumor cell lines infected with RCAS-Neu/Ctr-miRNA or RCAS-Neu/mHPR1-miRNA 746 were seeded in 96-well plates at the density of 2,000/well. After incubation for 96 hr, cell proliferation was monitored by using an ATP-based luminescence assay (Promegan, Madison, WI) following the manufacturer's instruction.

FACS analysis of cell surface HS. RACS-Neu and DF-1 cells transfected or infected with various RCAS vectors were harvested and analyzed for cell surface HS levels by staining with an anti-HS mAb (clone HepSS) followed flow cytometry according to previous publications [26].

***In vivo* tumor induction.** TVA-transgenic mice expressing the receptor for an avian retrovirus vector, RCAS, were infected with DF-1 cells transfected with RCAS-PyMT vector, RCAS-Neu/mHPR1-miRNA, RCAS-Nue/LacZ-miRNA, RCAS-8C by intraductal injection. Mice infected with RCAS-PyMT virus were treated with sulodexide by i.p. injection. Mice were observed for breast cancer development by palpation. The differences of tumor latency between

untreated and sulodexide-treated groups, RCAS-Neu/mHPR1-miRNA versus RCAS-Neu/LacZ-miRNA, RCAS-Neu plus RCAS-8C versus RCAS-Neu plus RCAS-GFP group, were statistically analyzed by using the Log-rank test. The difference of tumor growth between three groups was statistically analyzed by using the one-way repeated measure ANOVA. The p value of <0.05 was considered statistically significant.

Results

Sulodexide treatment accelerates PyMT-mediated tumorigenesis. Sulodexide is a mixture of dermatan sulfate (20%) and low-molecular-weight heparin (80%). We first examined the ability of sulodexide to inhibit HPR1 activity by using a novel ELISA method developed in my laboratory [24, 25, 27-29]. As shown in Fig. 1A (left panel), sulodexide inhibited HPR1 activity with an IC_{50} value of approximately 5 μg/ml. The IC_{50} values for heparin and PI-88 were approximately 2-3 μg/ml. Sulodexide inhibits HPR1 activity slightly better than PI-88 and heparin when a pancreatic cancer patient's serum was used as the source of HPR1 (Fig. 1, right panel).

We next tested whether sulodexide can prevent breast tumor formation. TVA transgenic mice were infected by intraductal injection of RCAS-PyMT virus, $1x10^7$ virions/gland, 4 glands/mouse. Mice were treated with water or sulodexide at the dose of 35 or 70 mg/kg/day by gavage and monitored for tumor formation by palpation. To our surprise, administration of sulodexide accelerated breast cancer formation in a dose-dependent manner (Fig. 2). Breast tumors were formed in untreated mice with the median latency of >78±4.1 days, whereas administration of sulodexide at 70 and 35 mg/kg/day had a median tumor latency of 23±2 days and 53±7.4 days, respectively. Log-rank test showed that sulodexide treatment at the dose of 35 mg/kg/day significantly shortened tumor latency, compared to the untreated control group ($p=0.018$). Also, Log-Rank test revealed that mice treated with sulodexide at 70 mg/kg/day had a significantly shorter tumor latency than those treated with sulodexide at the dose of 35 mg/kg/day ($p=0.002$).

Effect of HPR1 gene knockdown on breast cancer tumor growth. The role of HPR1 in breast tumor growth and tumorigenesis was further tested by using a genetic approach. Three miRNA constructs were prepared by using a hairpin sequence that target murine HPR1 mRNA at the nucleotide site of 671, 746, and 796. As shown in Fig. 2A, FACS analysis revealed that cell surface heparan sulfate levels were decreased in RCAS-Neu/mHPR1-miRNA 746-infected cells, compared to that infected with RCAS-Neu/LacZ-miRNA. Western blot analysis confirmed the ability of this vector to suppress HPR1 expression in RCAS-Neu breast cancer cell lines (Fig. 2B). HPR1-miRNA fragment was also cloned into the downstream of RCAS-PyMT vector. Similar results were obtained with RCAS-PyMT/HPR1-miRNA construct (data not shown).

In vitro study showed that knockdown of HPR1 expression in RCAS-Neu cells did not affect cell proliferation (Fig. 2C). We next examined the effect of HPR1 knockdown in tumor growth in a syngeneic mouse model. RCAS-Neu cells stably transfected with RCAS-Neu/HPR1-miRNA or the control construct RCAS-Neu/Ctr-miRNA were inoculated into the fat pad of FVB mice ($5x10^5$ cells per fat pad) (8-12 mice/group). As shown in Fig. 3, knockdown of HPR1 expression led to a significant suppression of the growth of RCAS-Neu tumor cells with HPR1 knockdown, compared to that transfected with RCAS-Neu/LacZ-miRNA.

The effect of HPR1 knockdown on tumor initiation. RCAS-Neu/HPR1-miRNA and RCAS-Neu/Ctr-miRNA retroviral vectors ($1x10^7$ virions/gland) were used to induce breast cancer by intraductal injection into the mammary gland of TVA transgenic mice carrying the transgene encoding the receptor for the sub-group A avian leucosis virus. As shown in Fig. 4, mice infected with RCAS-Neu/HPR1-miRNA developed breast cancer significantly slower than those infected with RCAS-Neu/Ctr-miRNA.

The effect of the C-terminus of HPR1 on breast cancer formation. Three RCAS vectors containing a full-length HPR1 gene, an enzymatic activity-dead HPR1 gene (RCAS-DM-

HPR1, double mutations at amino acid residues 225 & 343), and a C-terminus gene fragment (RCAS-8C, with a fusion of 8-kDa and the C-terminus of HPR1 including amino acid residues from 415-543) were used to transfect DF-1 cells. Western blot analysis with an anti-Myc tag antibody revealed that HPR1 was detected as an 18-kDa protein in DF-1 cells transfected with RCAS-8C vector, whereas the full-length HPR1 was detected as 50-kDa protein. Immunofluorescence staining revealed that RCAS-HPR1 virus-infected DF-1 cells had lower cell surface heparan sulfate levels, compared to RCAS-8C-infected DF-1 cells. These results confirmed that the C terminus of HPR1 did not have HPR1 enzymatic activity.

To determine whether the C-terminus of HPR1 was able to promote tumor initiation, TVA mice were co-infected with RCAS vectors encoding the RCAS-Neu virus plus the control RCAS vector or the vector encoding RCAS-8C. As shown in Fig. 5C, mice infected with RCAS-Neu plus RCAS-8C vectors developed breast cancer much faster than those infected with a control RCAS-GFP vector, the mean and median tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-8C were 275±27 and 180±30 respectively, whereas the mean tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-GFP were 480±25 and 245 respectively. These observations strongly suggested that that HPR1 enzymatic activity is not necessary for its tumor promoting effect.

Discussion

Conventional transgenic mice carrying the PyMT or rat Neu proto-oncogene in their genomes are genetically predestined to overexpress Neu or PyMT in all mammary epithelial cells and to develop lethal invasive mammary carcinomas [30]. Our studies using a clinically relevant breast cancer model to investigate the role of HPR1 on tumor formation. In this mouse model, the expression of Neu or PyMT onconge is restricted to a few mammary epithelial cells. Therefore, our mouse model closely resembles a situation in patients in which Neu-positive breast cancers also originate from a few cells. Our results demonstrated the HPR1 knockdown slowed down the formation of Neu oncogene-induced breast cancer (Fig. 4), whereas co-infection with the C-terminus of HPR1 with the Neu oncogene accelerated breast cancer induction (Fig. 5). These results collectively suggest that HPR1 is able to promote tumor formation.

Our studies also suggested that HPR1 enzymatic activity is disposable for its tumor promoting activity. Using a HRP1 inhibitor, our study demonstrated that sulodexide was not only unable to slow down breast cancer initiation but rather could accelerate tumorigenesis initiated by a PyMT oncogene. There are several plausible explanations for these unexpected results: 1) Our recent in vitro study showed that HPR1 inhibitors are able to stimulate the proliferation of pancreatic cancer cells by increasing the expression of cell surface HPSGs and strengthening the FGF2 receptor-activated MAP kinase pathway [25]. It is possible that sulodexide may stimulate the proliferation of PyMT-transformed breast cells in vivo by a similar mechanism; 2) HSPGs function as the co-receptor for Wnt and FGF-2, both growth factors are involved in stem cell self-renewal [31, 32]. It is possible that increased cell surface HSPG levels by sulodexide may enhance the FGF signaling pathway, leading to the acceleration of breast

cancer formation. These observations are consistent with previous observations that enzymatically inactive HPR1 is more efficient in stimulating tumor growth [13, 14]. Thus, these results collectively suggest that HPR1 enzymatic activity is not only disposable but rather may negatively regulate tumor growth. The strategy using HPR1 inhibitors to target its enzymatic activity may not work because of the potential to HPR1 inhibitor to stimulate tumor growth.

In the present study, we also investigated the role of HPR1 in tumor growth. Using a syngeneic breast cancer model, we found that HPR1 knockdown led to a retarded tumor growth (Fig. 3). These results were not unexpected since several prior studies using xenograft mouse model demonstrated slower growth of implanted human breast cancer cell lines with HPR1 knockdown, compared to their control vector-transfected cell line controls. Interestingly, in vitro cell proliferation experiments revealed that HPR1 knockdown did not lead to an inhibition of cell proliferation. This observation is consistent with a prior study showing that HPR1 overexpression in a human breast cancer cell line MDA-MB-231 did not increase cell proliferation in vitro but increase breast tumor growth in a nude mouse breast cancer model by stimulating tumor angiogenesis[12]. It appears that HPR1 knockdown-mediated antitumor tumor effect is likely mediated by its effect on tumor angiogenesis in our mouse model. Notably, Cohen-Kaplan et al. [19] reported that HPR1 overexpression is able to increase the proliferation of a LNCaP human lung cancer cell line, whereas HPR1 knockdown leads to decreased DNA replication in a MDA-231 breast cancer cell line and U87 glioma cell line, assuming that the cell proliferation is also inhibited in these HPR1-suppressed cell lines. The discrepancy in the effect of HPR1 on cell proliferation is not clear.

We noticed that there was some discrepancy in tumor induction in different experiments. One explanation could be due to the success rate of virus injection into the mammary gland. Second possibility could be due the discrepancy in the virus stock prepared at different time and

storage. One weakness in the present study is that the effect of full-length HPR1 on breast cancer formation has not been tested (data unavailable now).

In summary, our present study demonstrated that HPR1 is able to stimulate breast tumor growth and to accelerate breast cancer initiation in a clinically relevant breast cancer model. The enzymatic activity of HPR1 is disposable for the stimulatory effect of HPR1, whereas the C-terminus of HPR1 plays a critical role in promoting breast cancer initiation.

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We are very grateful to Dr. Yi Li (Baylor College of Medicine) for providing murine breast cancer cell lines, RCAS-GFP, RCAS-Neu and RCAS-PyMT vectors, and the TVA transgenic mouse line. The authors also thank Keryx Pharmaceuticals Inc. (New York, NY) for providing sulodexide. This project was supported U.S. Army Breast Cancer Research Program (W81XWH-11-1-0302) to X. Xu.

Figure legends

Fig. 1. Inhibition of HPR1 activity by PI-88, heparin and sulodexide. Purified platelet HPR1 (50 units/μl) (A) or serum (B) from a pancreatic cancer patient diluted at 1:10 in HPR1 buffer were premixed with the indicated concentrations of HPR1 inhibitors in HPR1 assay buffer. The mixture was added to a 96-well ELISA plate precoated with Matrigel and incubated at 37°C for 16 hr. HPR1 activity was analyzed by an ELISA method. (C) Sulodexide treatment accelerates PyMT tumor formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-PyMT virus by intraductal injection of $1x10^7$ virions, 4 glands per mouse. One week later, mice were treated daily with water (20 mice), sulodexide at the dose of 35 mg/kg/day (18 mice) or 70 mg/kg/day (9 mice) by gavage. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test.

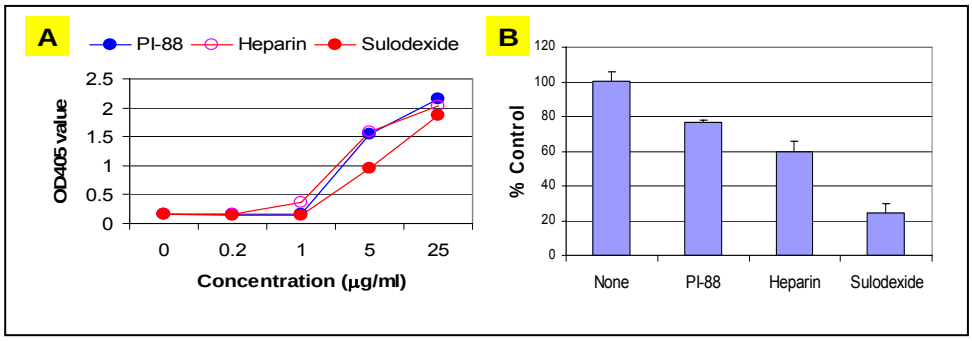
Fig. 2. Increased cell surface HS levels by mHPR1 knockdown. RCAS-Neu cells were infected with RCAS-Neu/Ctr-miRNA (left), RCAS-Neu/mHPR1-miRNA-746 (right). After incubation for 48 hr, the cells were harvested, stained with an anti-HS IgM mAb, analyzed for cell surface HS levels by FACS analysis (A) or for HPR1 expression by Western blot (B). Black line, isotype control; Green line, anti-HS IgM. (C) HPR1 suppression does not inhibit tumor cell proliferation. RCAS-Neu cells infected with RCAS-Neu/LacZ-miRNA or RCAS-Neu/HPR1-miRNA virus were seeded in 96-well plates (2000 cells/well) and incubated for 24 or 72 hr. Cell proliferation was analyzed by an ATP-based Cell-Glo assay and read in a 96-well plate reader. The data represents the mean standard deviation of one of three experiments in triplicate with similar results.

Fig. 3. HPR1 knockdown suppresses breast tumor growth. Female FVB mice (8-12 weeks old) were inoculated with RCAS-Neu/HPR1-miRNA or RCAS/LacZ-miRNA cells by fat pad injection of $5x10^5$ cells. Mice were monitored for tumor growth 3 weeks later and measured twice weekly with a caliper. The difference of tumor growth between three groups was statistically analyzed by using the one-way repeated measure ANOVA.

Fig. 4. HPR1 knockdown delays breast cancer formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-Neu/HPR1-miRNA or RCAS-Neu/LacZ-miRNA virus by intraductal injection of $1x10^7$ virions. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test ($p<0.01$)

Fig. 5. HPR1 expression and cell surface HS levels. DF-1 cells were infected with RCAS-HPR1 (left), RCAS-DM-HPR1 (middle) or RCAS-HPR1-8C. After incubation for 48 hr, the cells were harvested and analyzed for HPR1 expression by Western blot with an anti-Myc epitope antibody (A) or for cell surface HS levels by staining with an anti-HS IgM mAb followed by FACS analysis (B). Black line, isotype control; Green line, anti-HS IgM. (C) HPR1 knockdown delays breast cancer formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-Neu plus RCAS-HPR1-8C (blue line) or RCAS-Neu plus RCAS-GFP virus (red line) by intraductal injection of $1x10^7$ virions each. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test ($p<0.01$)

Fig. 1


A
PI-88
Heparin
Sulodexide
OD405 value
2.5
2
1.5
1
0.5
0
0
0.2
1
5
25
Concentration (μg/ml)
B
% Control
120
100
80
60
40
20
0
None
PI-88
Heparin
Sulodexide

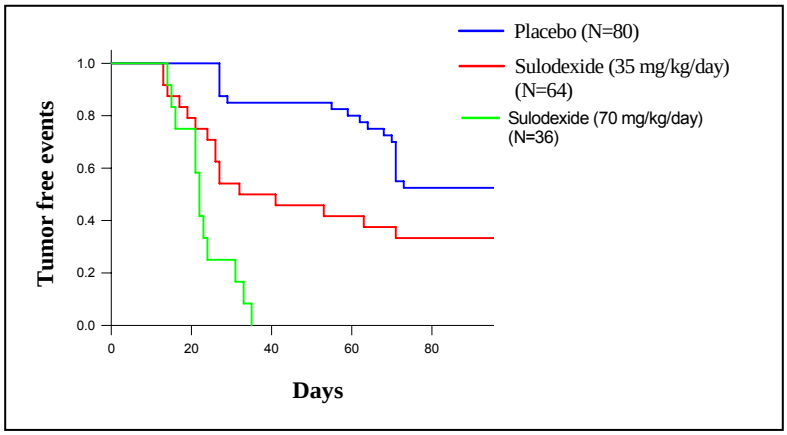

Placebo (N=80)
Sulodexide (35 mg/kg/day) (N=64)
Sulodexide (70 mg/kg/day) (N=36)
Tumor free events
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Days

Fig. 2.

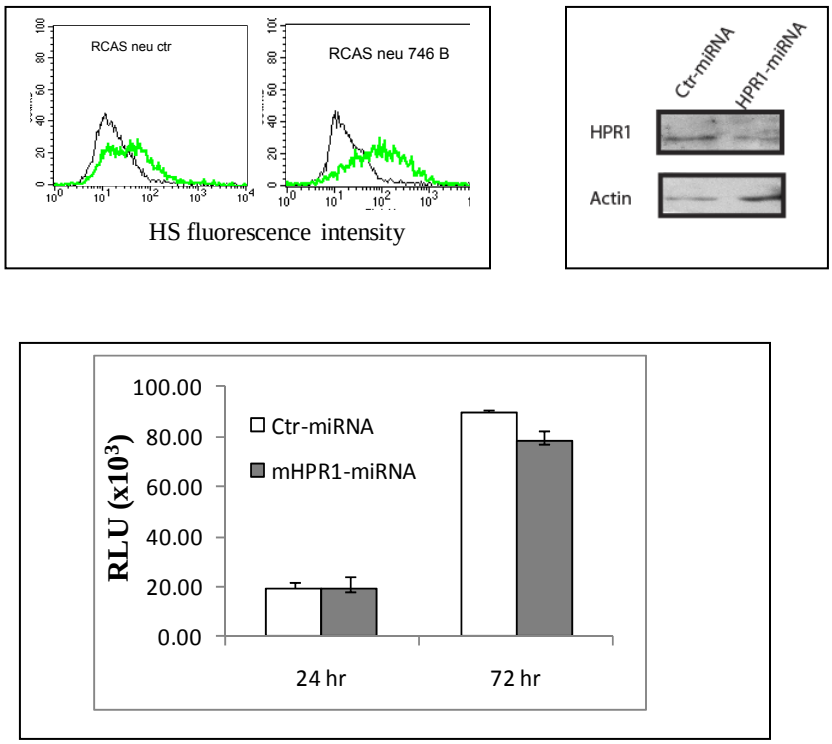

RCAS neu ctr
RCAS neu 746 B
HS fluorescence intensity
Ctr-miRNA
HPR1-miRNA
HPR1
Actin
RLU (x10³)
100.00
80.00
60.00
40.00
20.00
0.00
Ctr-miRNA
mHPR1-miRNA
24 hr
72 hr

Fig. 3

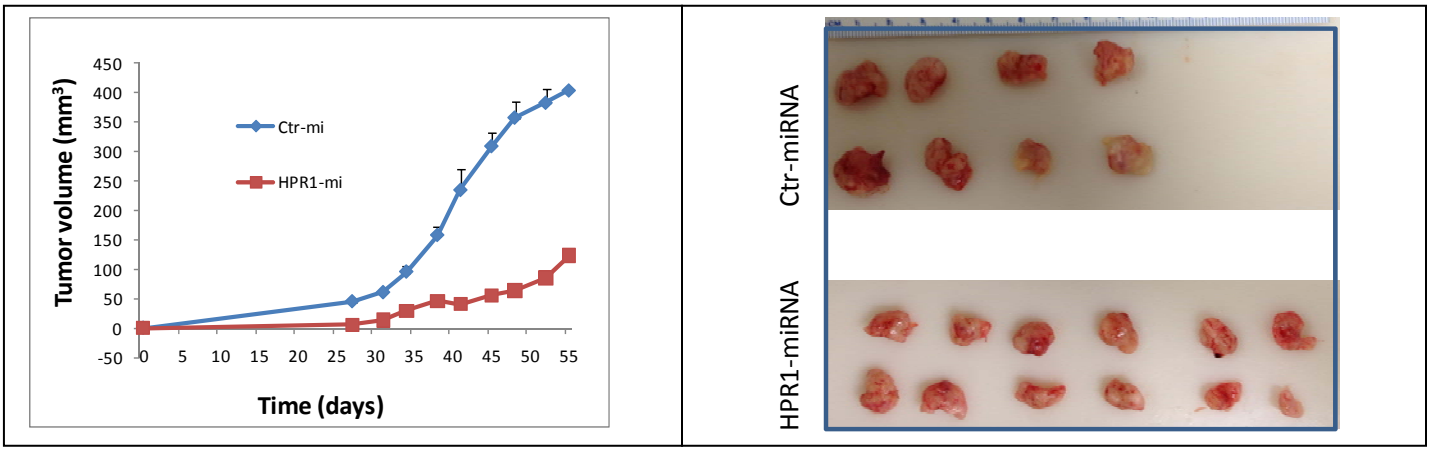

Tumor volume (mm³)
450
400
350
300
250
200
150
100
50
0
-50
0 5 10 15 20 25 30 35 40 45 50 55
Time (days)
Ctr-mi
HPR1-mi
Ctr-miRNA
HPR1-miRNA

Fig. 4

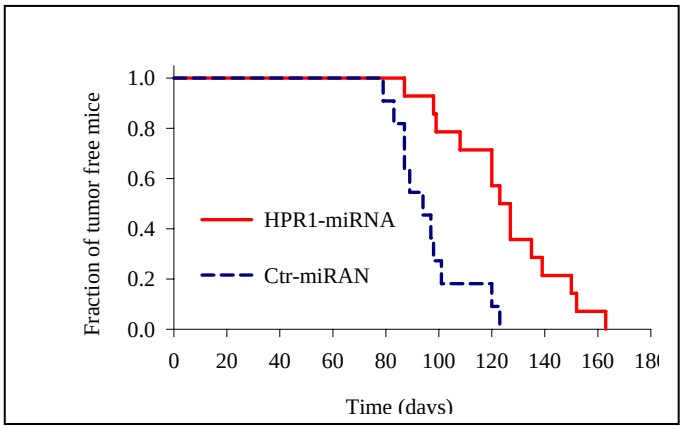
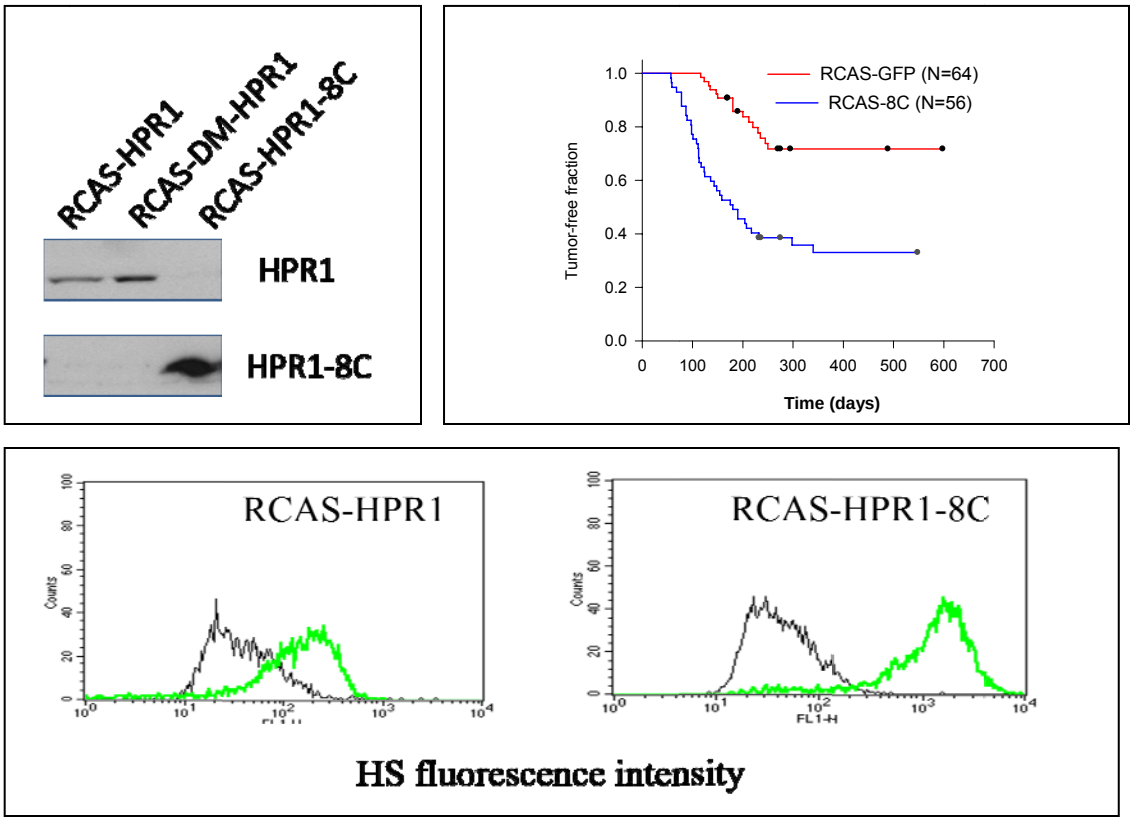

Fraction of tumor free mice
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80 100 120 140 160
Time (days)
HPR1-miRNA
Ctr-miRAN

Fig. 5


RCAS-HPR1
RCAS-DM-HPR1
RCAS-HPR1-8C
HPR1
HPR1-8C
RCAS-GFP (N=64)
RCAS-8C (N=56)
Tumor-free fraction
Time (days)
RCAS-HPR1
RCAS-HPR1-8C
Counts
FL1-H
HS fluorescence intensity

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